REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
23-02-2011	Journal Article	

4. TITLE AND SUBTITLE

Measuring the Variability of Treated Bacillus anthracis delta Stern Spores

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

ECBC, Aberdeen Proving Ground, MD
Battelle Memorial Institute, Aberdeen Proving Ground, MD
Science Applications International Corporation, Abingdon, MD
Science and Technology Corporation, Edgewood, MD

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Distribution Statement: Approved for Public Release; Distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The development of biological detectors for the military and homeland defense 26 relies on the ability to test hardware with biological materials that can be produced reliably 27 and reproducibly. To ensure occupational safety during the development of new devices, 28 the production and testing with biological materials necessitates compromises that include 29 selection of avirulent strains and inactivation using various techniques. The materials and 30 methods selected for production and post-production treatment can greatly influence 31 detection outcomes using sensitive DNA or antibody based systems. The optimal situation 32 would be to reproducibly create biological testing materials that accurately simulate the 33 true pathogen yet carry minimal risk to the scientists and engineers developing the 34 biosensors. In this study, avirulent Bacillus anthracis spores were used to demonstrate the 35 varied outcomes that can be induced by density gradient purification and post-growth 36 inactivation using gamma irradiation. The responses using Quantitative Polymerase Chain 37 Reaction, Electrochemiluminescence, Enzyme-Linked Immunosorbent Assay, 38 Bicinchoninic Acid assay, and protein electrophoresis varied significantly with treatment. 39 Gradient purification removed proteins that interfered with monoclonal and polyclonal 40 antibodies' abilities to form immunocomplexes in Electrochemiluminescent and ELISA 41 assays. Irradiation had a varied effect on qPCR depending on the sample preparation 42 employed and produced a reduced response in Electrochemiluminescent and ELISA 43 assays. Varied results from enumeration techniques show the importance of measuring the 44 dead cellular material and cellular debris contribution to any sample. Production and 45 material treatments need to conform to a yet to be established guideline that can bring 46 uniformity to available biological agent reference materials.

15. SUBJECT TERMS

Bacillus anthracis, detection, sensors, variability

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Bryan Horner
unclassified	unclassified	unclassified		34	**19b. TELEPHONE NUMBER** *(include area code)* 703.767.3379

Title: **Measuring the Variability of Treated *Bacillus anthracis delta* Sterne Spores**

Running Title: **Variability between Treatments of *Bacillus* Surrogates**

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The development of biological detectors for the military and homeland defense relies on the ability to test hardware with biological materials that can be produced reliably and reproducibly. To ensure occupational safety during the development of new devices, the production and testing with biological materials necessitates compromises that include selection of avirulent strains and inactivation using various techniques. The materials and methods selected for production and post-production treatment can greatly influence detection outcomes using sensitive DNA or antibody based systems. The optimal situation would be to reproducibly create biological testing materials that accurately simulate the true pathogen yet carry minimal risk to the scientists and engineers developing the biosensors. In this study, avirulent *Bacillus anthracis* spores were used to demonstrate the varied outcomes that can be induced by density gradient purification and post-growth inactivation using gamma irradiation. The responses using Quantitative Polymerase Chain Reaction, Electrochemiluminescence, Enzyme-Linked Immunosorbent Assay, Bicinchoninic Acid assay, and protein electrophoresis varied significantly with treatment. Gradient purification removed proteins that interfered with monoclonal and polyclonal antibodies' abilities to form immunocomplexes in Electrochemiluminescent and ELISA assays. Irradiation had a varied effect on qPCR depending on the sample preparation employed and produced a reduced response in Electrochemiluminescent and ELISA assays. Varied results from enumeration techniques show the importance of measuring the dead cellular material and cellular debris contribution to any sample. Production and material treatments need to conform to a yet to be established guideline that can bring uniformity to available biological agent reference materials.

Bacillus anthracis is a Category A agent and is the causative agent of the potentially fatal disease "anthrax" (9,7). *B. anthracis* is the gram-positive, non-motile, non-chemolytic, spore-forming bacteria that attracted renewed international attention when transported through the United States mail to members of Congress and the media in November 2001. These mailings and the increased threat from extremist groups have fueled a surge in biological agent sampling and detection defense research that accelerated after the 1991 Gulf War. Currently there are over 100 biological detectors and commercially available kits (17,14) with many more under development. The Department of Defense, Department of Homeland Security, and the commercial sector have been actively involved in supporting developmental testing of new biological agent detection equipment (13,3,20,21,25,18).

Developmental testing and use of biological detectors for environmental or diagnostic purposes requires the use of biological reference material to properly validate performance. Currently, researchers and instrument developers working to detect *B. anthracis* inadvertently build variability into their results by the use of near neighbor (5,19) *Bacillus* species (*B. cereus* group) or avirulent *B. anthracis* (29) to avoid occupational exposures, and by use of varied growth conditions (1) (media, environmental) and/or preparation procedures.

The development and use of chemical and radiological standards is a well established field with multiple reference materials offered for research and development operations around the world. In the arena of biological based standards and references, materials are not as standardized or as rigorously tested as their chemical and radiological counterparts due to the inherent variability of standardizing living material. Biological reference materials are available for purchase, including simulants of bio-warfare agents, but not necessarily marketed as a standard reference material. Because of the relative ease of production, these materials are

commonly used as seed stock to produce quantities required for specific testing. Therefore, in addition to variations that may be inherent between the reference materials obtained from multiple sources, the researcher may further compromise the integrity of their materials during their own in-house production and treatment. Additionally, small quantity research grade materials may be processed more stringently than larger productions, thus producing different responses upon presentation to a particular assay. It is also common practice for researchers and developers to use irradiation killed materials (8,30,28,27,2) to meet personnel and/or open air testing safety concerns.

The aim of this program was to establish standardized protocols for producing, processing, and analyzing biological reference materials in support of test and evaluation programs. As a result of these studies production methodology and conformance test plans are now under review by the science and technology departments within the Department of Defense agency which funded these studies. Department of Defense programs will benefit by the ability to produce reliable test materials in support of their mission objective to field next generation detection and diagnostic systems. Electrochemiluminescence (ECL), Enzyme-Linked Immunosorbent Assay (ELISA), Quantitative Polymerase Chain Reaction (qPCR) and Bicinchoninic Acid (BCA) assays are included as conformance tests to characterize biological test reference materials. In addition, this program attempted to demonstrate the response commonly used analytical instruments have when testing biological materials that have been prepared using common, but varied, lab practices. This paper describes and summarizes those in-house laboratory results.

The material used in this study, *Bacillus anthracis delta* Sterne, lacks both the pXO1 and the pXO2 plasmids (11) ($pXO1^-$, $pXO2^-$), and is a spore forming bacteria of the *B. cereus* group.

The absence of these two plasmids in the *B. anthracis delta* Sterne strain renders the material two steps removed from the lethal *B. anthracis* and provides spores with similar characteristics to other *B. anthracis* strains without the pXO2 plasmid markers. In this study, identically grown spores were subjected to three different, but common, forms of downstream processing. Two processes vary the degree of purification, a triple wash followed by gradient purification versus a single wash only preparation. The third process involved the triple wash plus gradient purification followed by a gamma irradiation kill step. These three preparations were then analyzed using quantitative PCR (qPCR), Electrochemiluminescence (ECL), ELISA, Bicinchoninic Acid protein assay and a protein electrophoresis microchannel assay.

MATERIALS AND METHODS

Cell/Spore preparation (G Media). *Bacillus anthracis delta* Sterne was obtained from the Unified Culture Collection # BACI056 (Critical Reagents Program, Edgewood, MD) and grown in nine 4L cultures of G-media plus trace metals at 37^{o}C until >95% sporulated. Percent sporulation was determined under phase contrast microscopy by visual count using differential spore stain (Schaeffer-Fulton 5% malachite green). Purity was verified during production by gram staining and phase contrast microscopy at points including seed stock verification, isolation streak, Nutrient Broth inoculum, 2X expansion, pre- and post-growth processing. Media was removed through centrifugation at 6,000 X *g* for 30 min at 4°C and after, three samples were designated as Ultra-Pure -1, -2, and -3; three as 1X Wash -1, -2, and -3; and three samples as Gamma-Irradiated -1, -2, and -3. The three 1X Wash pellets were washed once with 500 mL of 0.01M ice cold Phosphate Buffer Solution (PBS, Becton Dickinson Difco, Franklin Lakes, NJ), and centrifuged at 4,500 X *g* for 15 min at 4°C. The supernatant was decanted and

the pellets resuspended in 100 mL sterile ice cold 0.01M PBS, and one mL aliquots were prepared and stored at -80^{o}C until analyzed. The Ultra Pure and Gamma Irradiated samples were each washed 3 times and resuspended using the same procedures and volumes. The final washed pellet was resuspended in 100 mL of 70% ethanol (Acros, Morris Plains, NJ) and held at room temperature for 1 h at 150 rpm. The samples were centrifuged at 4,500 X *g* for 30 min at 4°C. After decanting the supernatant, the pellets were resuspended in 500 mL sterile 0.01M PBS, then heat shocked at 65°C for 1 hr at 150 rpm.

Gradient Purification. After heat shock, the samples were centrifuged at 4,500 X *g* for 15 min at 4°C. The triple washed, ethanol treated, and heat shocked final pellets were resuspended in a 20% preparation of Renografin-60 (Bracco Diagnostics, Princeton, NJ), overlaid onto a 1:5 ratio of a 50% preparation of Renografin-60 and centrifuged at 10,000 X *g* for 1 hr at 4°C. The supernatant was decanted and the pellets resuspended in 500 mL ice cold sterile 0.01M PBS. The samples were washed three times in 500 mL ice cold sterile 0.01M PBS centrifuged at 7,000 X *g* for 30 min at 4°C, and the supernatant decanted. After the final wash, the pellets were resuspended in 100 mL ice cold sterile 0.01M PBS. One mL aliquots of Ultra Pure -1, -2, -3 were prepared and stored at -80°C until analyzed. Gamma irradiated -1, -2, -3 were aseptically transferred to three sterile 50 mL conical tubes with 33.3 mL per tube. Spore suspensions were dosed to 50 kGy (5.0 x 10^{6} rads) using a Cobalt-60 gamma irradiation system (J.L. Shepard and Associates, Model 484R-2). One mL aliquots were stored at -80^{o}C until analyzed.

Killed *B. anthracis delta* Sterne spores were received from the CRP and used untreated as a reference material in Electrochemiluminescence and ELISA assays. Sample concentration was used as furnished as the referee.

Enumeration by Plating. Spore concentration (CFU/mL) for each growth was determined using an automated spiral plate technique (15,10), (AP4000 Spiral Biotech, Inc., and Q-Count Advanced Instruments, Norwood, MA) on TSA.

Enumeration by Flow Cytometry. Spiral plate enumeration results were complimented by the MicroPRO™ automated flow cytometer (Advanced Analytical Technologies, Inc. [AATI], Ames, IA). The MicroPRO, AATI's updated Rapid Biological Detector 3000 platform, uses differential staining to enumerate viable and non-viable organisms. AATI's Total Viable Organism assay kit was used to measure live cell counts which consists of a 3 min incubation of the test sample with 100 μL of a positively charged membrane permeable Nucleic Acid Dye (Molecular Probes, Inc., Eugene, OR) that binds to all living and dead cells. An 8 min incubation follows with 100 μL of BRAG3, AATI's patented non-membrane permeable counter stain that quenches the fluorescence of membrane-compromised cells, and extraneous debris, to ascertain a live cell count for Ultra Pure and 1X Wash treatments. Non-viable cells were counted using AATI's Dead Cell Assay, which consists of a 3 min incubation of the test sample with 100 μL of a positively charged non-membrane permeable dye that stains membrane compromised (non-viable) cells. Test samples were prepared in PB Buffer (AATI, Ames, IA) from stock using serial dilutions to 10^{-4} CFU/mL. Three hundred microliters of each dilution was then added to 2,700 μL of PB Buffer in a 12 x 75 mm culture tube and placed into the sample tray. Samples were treated with Total Viable Organism or Dead Cell stain and the sample was excited at 635 nm. Side scatter and fluorescent intensity measurement of the fluorochrome-labeled cells allowed for quantification of viable or non-viable cells in number/mL.

Quantitative Real-Time Polymerase Chain Reaction (qPCR); **Sample Preparation.** Three sample parameters were prepared for qPCR analysis; "Neat" consisted of the sample dilutions prior to centrifugation without treatment (mixed cellular and extra-cellular DNA), "Supernatant" (extra-cellular DNA) was the material removed from the cellular pellet following centrifugation, and FastPrep™ (cellular DNA, treated) was the pelletted and homogenized material collected from the FastPrep system.

To yield multiple data sets in the instruments calibration range sample stocks were diluted in PBS (Becton Dickinson difco, Franklin Lakes, NJ), pH 8, from 1.0×10^6 to 1.0×10^3 CFU/mL. One milliliter was pelleted by centrifugation at 14,000 X *g* for 5 min. The supernatant was removed and the pellet was resuspended in 1mL of PBS. The resuspended pellet (FastPrep) was added to Lysing Matrix E tubes (MP Biomedicals, Solon, OH) and homogenized in a FastPrep system (MP Biomedicals, Solon, OH) at 6.5m/s for 1.5 minutes.

Quantitative Polymerase Chain Reaction. Amplification, data acquisition, and analysis were carried out on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using a 96-well, standard block format. PCR reactions were performed in duplicate on 4-log serial dilutions. Each reaction well contained 5µL of sample and 15µL of Master Mix [Critical Reagents Program, Aberdeen Proving Ground, MD]. The Critical Reagents Program target three master mix assay for *B. anthracis* is a chromosomal target assay using proprietary primer and probe sequences. The reaction chemistry is optimized for the master mix on the ABI 7900 platform using the standard heating block. The thermocycling profile was: two min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C followed by 1 min at 60°C. Each reaction contained 1.25 U of Platinum *Taq* DNA polymerase that was provided by

185 the Critical Reagents Program. Quantitation was achieved by generating a standard curve from
186 parallel amplification reactions using known amounts (100 pg to 100 fg) of *Bacillus anthracis*
187 *delta* Sterne DNA (Critical Reagents Program, Aberdeen Proving Ground, MD) as template
188 material.

189 The data was analyzed using software provided with the ABI 7900 system. Using the
190 concentrations assigned to each DNA standard, the software constructed a standard curve of
191 Cycle threshold (*Ct*) value versus concentration. Concentration values for the unknown samples
192 were extrapolated from the standard curve by the software. All samples within the calibration
193 limits were normalized to the 1.0×10^6 CFU/mL sample concentration.

194 The CT value (n) was used to calculate the genome equivalent (GE). Genomic
195 equivalent is a value determined for a sample specimen using qPCR, numerically equivalent to
196 the copy number of the gene targeted for amplification, reported as a concentration (GE mL^{-1}),
197 which is correlated to concentration (CFU/mL). Genome equivalent is calculated by converting
198 chromosome size to mass using m = (n) ($1.096e^{-21}$ g bp^{-1}) where m = mass of chromosome, n =
199 number of nucleotide base pairs (bp), and the constant $1.096e^{-21}$ is derived from Avogadro's
200 number and the average mass of a nucleotide bp (650g mol^{-1}). One gene copy is present per
201 chromosome, which determines the GE by dividing the PCR determined mass of DNA (by
202 qPCR) by the chromosome size (mass).

203 **Protein Quantitation.** Protein quantitation was performed using the Pierce BCA™
204 Protein Assay Kit (Pierce, Rockford, Illinois; Catalog #23225). Bovine Serum Albumin was
205 diluted in 0.01M PBS to concentrations ranging from 1500 to 0 µg/mL to construct a standard
206 curve. Test samples were diluted serially in 0.01M PBS from stock to a concentration of
207 1.0E+05 CFU/mL. Bicinchoninic acid (BCA) analysis of samples was performed following the

protocol outlined by Smith (26) with 200 µL of BCA working reagent added to 25 µL of sample in a microtiter plate, followed by a 30 min incubation at 37^{o}C. Samples were read and amount of protein quantified at 562 nm using a VersaMax Microplate Reader (MDS Analytical Technologies, Sunnyvale, CA).

Microfluidic Protein Determination. To determine any presence of extractable antigen 1 (EA1) protein (~100kDa), supernatant from each growth treatment was run on the Agilent 2100 Protein 230 LabChip according to manufacturer protocol. The Protein 230 LabChip provides data regarding the purity, concentration, and size of proteins within the sample through electrophoresis driven microchannels. A stock sample from each treatment was pelleted at 10,000 X *g* for 5 min at room temperature and 4 µL of the supernatant was transferred to a clean, 0.5 mL microcentrifuge tube. The samples were denatured following the manufacturer's protocol. The denatured supernatant was added to the wells on the LabChip in duplicate. The LabChip was run on the Agilent Bioanalyzer according to the settings installed by the manufacturer.

Electrochemiluminescence. Electrochemiluminescence analysis was performed using the BioVeris M1M Analyzer (BioVeris Corporation, Gaithersburg, MD). Test samples were diluted serially in 0.01M PBS + 0.05% Triton X-100 from stock to concentrations ranging from 1.0 x 10^{7} to 1.0 x 10^{3} CFU/mL. Initially, 25 µL of biotinylated antibody (prepared in-house using Molecular Devices Corporation's ImmunoLigand Biotin Label, Prod. # R9002), 25 µL of ruthenium-labeled antibody (prepared in-house using TAG NHS-ester™; BioVeris, Cat. # 110036), and 100 µL of sample dilution were combined in 0.75 mL assay tubes placed in a 96-well MINITube holder, and shaken for 10-min at 400 rpm. After shaking, 20 µL of Dynabeads

M-280 Streptavidin (Dynal, Cat. # 112.05D) at 1 mg/mL was added to the immunocomplex and Electrochemiluminescent measurements taken in triplicate

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA samples followed the same dilution scheme used for Electrochemiluminescence analysis. One hundred microliters of capture antibody, diluted to the appropriate concentration in Kirkegaard and Perry Laboratories (KPL) 1X ELISA coating solution (prepared from 10X solution), was added to a 96-well Immulon 2 HB U-bottom microtiter plate. The plate was incubated for 1hour in a VorTemp Plate Shaker Incubator at 37°C and 400 rpm. After incubation, the plate was washed three times with Abd Serotec 1X ELISA wash buffer using a TECAN 96-well plate washer (TECAN Inc., Cat. # 16029015). Two hundred microliters of KPL 0.1% milk blocking solution, prepared from 2% solution, was added to each well and incubated for 1 hr in the shaking incubator. The plate was then washed three times and 100 µL of the appropriate antigen concentration diluted in 0.1% milk blocking solution, was added to the wells and incubated for 30 min in a shaking incubator. The plate was washed three times, and 100 µL of the secondary antibody, diluted to the appropriate concentration in 0.1% milk blocking solution, was added to the wells and incubated for 30 min in the shaking incubator. The plate was washed three times and 100 µL of the appropriate anti-species Horse radish Peroxidase-labeled conjugate (KPL), diluted at 1:1000 in 0.1% milk blocking solution, was added to the wells and incubated for 30 min in the shaking incubator. The plate was then washed three times and 100 µL of KPL ABTS substrate (Cat. # 50-62-00) was added to the plate and incubated at ambient room temperature on an orbital plate shaker for 2 min at 500 rpm. After the 2 min shake, 100 µL of KPL 1X Stop Solution (prepared from 5X solution, Cat. # 50-85-01) was added to each well. The Optical Density at 405 nm was read on a VersaMax Microplate Reader.

Statistical Analysis. Results that fell outside of each instrument's calibration range and non-detects were eliminated. Standard curves for replicate analysis were examined for consistency (not shown). Each assay included positive and negative controls (not shown) to confirm proper instrument/assay operation. Each analysis was performed in triplicate at multiple concentrations when appropriate. Replicates within a given sample set across multiple dilutions were normalized to a standard concentration (e.g., 1.0×10^6) and combined for statistical analysis yielding at minimum 9 observations per set after disqualifications. Statistical outliers were eliminated using the Grubbs test (6, 16). Results for each treatment consisting of three operators for each individual growth were pooled and analyzed for mean, standard deviation, standard error, and confidence limits. Single factor Analysis Of Variance between treatments and graphical representations were accomplished using Microsoft Excel. Statistical differences were considered significant at $p<0.05$ using single factor Analysis of Variance.

RESULTS

Enumeration Plating. The three different preparations of spores were enumerated using dilution plating immediately before CTP analysis. The mean colony counts for each growth, presented in Table 1, were provided as the reference concentration for all CTP's for dilution and subsequent calculations. Spore concentrations for Gamma Irradiated growths were enumerated prior to the irradiation process and are provided here as a reference. Post Gamma Irradiated samples were spiral plated without dilution; no cell growth was observed.

Enumeration Flow Cytometry. Each different spore preparation was analyzed using the MicroPRO for contributions of live versus dead material contribution per sample. Comparison of results for combined post production treatments (Figure 1) indicates the Ultra Pure and 1X Wash Dead Cell count is a relatively low percentage (12-15%) of Total Viable

Organism. For Gamma Irradiated samples, the Dead Cell value represents the entire compliment of cells in the sample. The dead cell concentration, while a significant contribution to each of the purification treatments, does not indicate a significant difference between treatments. The irradiated treatment dead cell contribution is significantly different.

Comparison of MicroPRO and AP4000 Data. The MicroPRO and AP4000 both measure cell concentrations in a diluted suspension and are therefore open to comparison even though their methods of arriving at their respective values differ. The MicroPRO can count single cells it determines to be viable through differential staining, but cannot assure that the cells are the target organism, or that the cell it designates as viable would in fact grow when presented with appropriate growth conditions. A significant difference is noted between the Irradiated Dead Cell and the spiral plate results observed in Figure 2.

Quantitative Polymerase Chain Reaction. Three preparations of each spore treatment were prepared to observe differences in extra-cellular DNA contributions as a function of spore treatment. Sample limit of detection was established nominally at 1.0×10^3 CFU/mL based on cycle time of 0.05 pg calibration standard. Genome Equivalents of all dilutions was normalized to results of the 1.0×10^6 CFU/mL dilutions and results pooled by treatment are presented in Figure 3.

The FastPrep qPCR procedure is the high efficiency, baseline preparation for our qPCR samples. Genome Equivalents results for Ultra Pure indicate that unprocessed (Neat) samples of strictly cellular (spore) and extracellular DNA (sup) represent only 5.42 and 4.26 percent, respectively, of the FastPrep mixed DNA sample, indicating Genome Equivalents yield for non-FastPrep prepared spores is low, around 5 percent. Conversely, the cellular (spore) and extracellular DNA for the 1X Wash treatments represents 60.7 and 49.0 percent, respectively, of

301 the FastPrep mixed DNA sample. This indicates a much higher Genome Equivalents yield for
302 non-FastPrep prepared qPCR samples likely due to the ease of measuring DNA in the 1X Wash
303 samples containing a greater percentage of vegetative cells and extra-cellular DNA. An
304 interesting comparison is that of the Ultra Pure versus Gamma Irradiated "Neat" (cellular)
305 Genome Equivalents. The irradiation treatment apparently allows DNA to be more easily
306 extracted from intact spores (Neat, 16.4 %) without increasing extra-cellular (Supernatant,
307 4.87%) DNA, indicating that the spores may be weakened by irradiation, though not ruptured.
308 Ideally the FastPrep results for each post growth treatment should be the same. Analysis
309 of Variance results of FastPrep preparations indicate significant differences (P= 0.0027) between
310 Ultra Pure and 1X Wash samples. Other FastPrep comparisons, Ultra Pure vs Gamma Irradiated
311 and 1X Wash vs Gamma Irradiated are not different. Significant differences exist between Ultra
312 Pure, 1X Wash, and Gamma Irradiated post growth culture treatments using the Neat qPCR
313 sample treatment. Supernatant preps of the Ultra Pure and 1X Wash treatments are significant,
314 while differences between Ultra Pure and Gamma Irradiated are not. The number of
315 observations ranged from 42 (Supernatant/Gamma Irradiated) to 74 (FP/Ultra Pure).
316 **Protein Determination**. Results for Bicinchoninic Acid protein varied widely at lower
317 concentrations prompting a search for a sample concentration level that yielded consistent
318 results. Reliability using this assay on *B. anthracis delta* Sterne spores began at a protein
319 concentration of approximately 15-µg/mL. No matter which post growth treatment tested, the
320 Bicinchoninic Acid assay on *B. anthracis delta* Sterne spores was not reliable below a sample
321 threshold of 1.0×10^7 CFU/mL. There is no effect of treatment on data reliability. Data from the
322 serial dilutions were normalized to 1.0×10^7 CFU/mL for comparison and are represented in
323 Figure 4.

Bicinchoninic Acid Analysis of Variance results indicate a significant difference in protein concentrations between "1X Wash" and the two other post growth treatments. This demonstrates the effect of decreased post growth washings and added renografin purification on total protein available to this assay. There was no difference between "Ultra Pure" and "Gamma Irradiated" treatments, indicating gamma irradiation of Ultra Pure spores in suspension had no effect on protein results for the assay used.

Microfluidic Protein Determination. The Protein 230 LabChip reveals a band at approximately 100 kDa in the Ultra Pure and 1X Wash treatments samples (Figure 5). The band is not present in the irradiated samples, owing to the destructive process of gamma irradiation. The 100 kDa band observed is likely due to the presence of the EA1 (extractable antigen 1) protein, or its closely related S-layer protein, SAP (surface array protein).

ElectroChemiluminescent Assay. Typical test sample concentrations ranged from 1.0 x 10^3 to 1.0 x 10^7 CFU/mL as refereed by AP4000. Net assay results in Electrochemiluminescent units are presented after subtraction of background (Figure 6). Response was linear through 1.0 x 10^6 CFU/mL with a limit of detection at 1.0 x 10^4 CFU/mL as mean background plus 3X Std-D for all samples.

Analysis of Variance was conducted for all treatments at 1.0 x 10^5 CFU/mL sample concentration, well within the linear response range for all samples. Differences were measured between Ultra Pure and all other samples, as well as 1X Wash and all other samples. No differences were observed between the Gamma Irradiated and Critical Reagents Program reference material. A decreased response (about half) was observed in the 1X Wash treatment samples compared to Ultra Pure samples. This decreased response in 1X Wash treatment is likely the result of higher EA1 and/or SAP protein content resulting from the decreased degree of

post growth purification compared to the Ultra Pure Treatment. The response to "Gamma Irradiated" and Critical Reagent Program samples are significantly lower than Ultra Pure and 1X Wash samples, but not significantly different from each other. The comparison between "Ultra Pure" and "Gamma Irradiated" treatments indicates irradiation has a significant effect on a sample's Electrochemiluminescent assay response.

ELISA. The Ultra Pure samples yielded the highest response while the Critical Reagents Program Reference material showed the lowest. The Ultra Pure samples showed the highest response with an OD_{405} above 2.0 with 1X Wash, the next highest, at 1.5 for sample concentrations of 1.0×10^6 CFU/mL (not shown). Gamma Irradiated and Critical Reagents Program samples did not illicit a response above OD_{405} of 1.0 Ultra Pure to 1.0×10^6 CFU/mL.

Response was linear through 1.0×10^5 CFU/mL with a limit of detection at 1.0×10^4 CFU/mL for Ultra Pure and 1X Wash samples, and a limit of detection at 1.0×10^5 CFU/mL for Gamma Irradiated samples. Analysis of Variance between treatments used data that was clearly in the linear range for all samples (1.0×10^5 CFU/mL, Figure 7). Analysis of Variance at 1.0×10^5 CFU/mL indicated differences between Ultra Pure and all other samples, as well as 1X Wash and all other samples, similar to the patterns that were observed in the Electrochemiluminescent assay data. No differences were observed between the Gamma Irradiated and Critical Reagents Program reference material. The greatest difference observed was between the Ultra Pure and Gamma Irradiated treatments. From Figure 7, the 1X Wash treatment's Optical Density response is observed to be significantly lower than the Ultra Pure treatment's response most likely resulting from higher EA1 or SAP protein content due to less post growth purification of the target *B. anthracis delta* Sterne spores. All growth treatments had a linear response up to a

sample concentration of 1.0 x 10^5 CFU/mL with goodness of fit (R^2) above 0.99. Change in response became non-linear at Optical Density above 1.0.

DISCUSSION

Enumeration. Dilution plating of bacteria is considered a standard for enumeration and was used to determine the referee concentrations used in our Conformance Test Plan testing, it however cannot account for non-living contributions in a sample. Dilution plating confirmed a 100% gamma irradiation kill rate in a 33 mL suspension. This is consistent with earlier studies (8,7) that found 25 Gy and 28 Gy were sufficient and our data adds to kill rates for larger volumes. From Figure 2, comparison of plating and MicroPRO™ Total Viable Organism and Dead Cell results indicate these methods agreed well with plating of Ultra Pure and 1X Wash treatments. Dead Cell analysis used for Gamma Irradiated samples did not agree with pre-irradiation plating results. The qPCR FastPrep results (Figure 3) validate the AP4000 enumeration results over the MicroPRO dead cell analysis. The Total Viable Organism vs Dead Cell results for live samples is valuable for measuring any contribution of dead organisms (Figure 1) to an analytical technique, like qPCR, that doesn't differentiate between live or dead. According to MicroPRO analysis, the dead component of Ultra Pure and 1X Wash samples are consistent from a low of 12.2% for 1X Wash-3 to a high of 15.5% for Ultra Pure-1 (not shown). Sporulated bacteria have long been known to be more resilient (4) than vegetative forms. Left-over, unaccounted for, dead vegetative material in a sample can induce a significantly different response under analysis. Determining this proportion mples considered live is an important component of sample validation.

Determination of Genomic Equivalents. Results of qPCR analysis shown in Figure 3 clearly show differences associated with post growth sample treatment and qPCR preparation.

The relative ratios of Neat and Supernatant GE are indicative of post growth purification. Extracellular DNA make up the majority of the GE measured in the Neat or no processing sample. The relatively small difference between the Neat and Supernatant samples once processed with FastPrep make up the entire cellular DNA GE response. The Neat and Supernatant preps of the qPCR 1X Wash sample Genome Equivalents are significantly higher than Ultra Pure and Gamma Irradiated samples. The elevated levels of cellular (pelleted) DNA (grey, figure 3), analyzed without qPCR pretreatment (beating or extraction), are indicative of non-spore, vegetative carryover from limited washings; fractured or poorly developed spore coats; and to a lesser degree, fully formed spores. The Supernatant (crossed, figure 3) pretreatment represents only extra-cellular DNA that is difficult to remove requiring additional washings or gradient purification. Differences in Neat prep results between Ultra Pure and Gamma Irradiated samples indicate spores are further weakened or fractured by γ-irradiation, thus allowing quantitation as an untreated preparation. Differences between Ultra Pure and Gamma Irradiated Supernatant preparations are not significant, indicating γ-irradiation treatment may weaken or fracture spores but do not produce added extracellular DNA.

Our comparison of the Gamma Irradiated vs non-Gamma Irradiated FastPrep samples disagrees with Daupin (8) who suggests that irradiation decreases the limit of detection using real-time PCR. Our data indicate no change in GE between the Ultra Pure and Gamma Irradiated FastPrep samples. Our data do indicate increases in available cellular DNA when no PCR pre-treatment is used, suggesting the previous studies (8) method of no pre-treatment of spore samples was inefficient and irradiation, a pre-treatment in this case, increases that efficiency. Daupin's (8) speculation that temperature control during irradiation affects *B. anthracis delta* Sterne detection limits is a valid concern that should be further addressed.

Protein Analysis. Analysis using the Bicinchoninic Acid method on spores indicated the assay is not particularly sensitive below a Limit of Detection of 1.0×10^7 CFU/mL. Data indicate significant protein carryover in 1X Washed samples. Gradient purification significantly lowered total protein content in Ultra Pure and Gamma Irradiated treatments. Comparison of Ultra Pure vs Gamma Irradiated samples show no significant difference. Total protein available to the assay is unaffected by irradiation treatment at the level of treatment used.

Protein 230 Chip. Chip data from Figure 5 indicate the presence of extractable antigen 1 (EA1) or surface array proteins (SAP) in Ultra Pure and 1X Wash post growth treatments. Both EA1 and SAP are known to be proteins attached to vegetative cells weighing approximately 100kDa. These proteins were not detected in Gamma Irradiated samples at the concentrations tested, indicating these proteins are destroyed during irradiation or at least lowered to undetectable levels. Limit of Detection is not known for these proteins using this assay. Our findings are consistent with those of Williams and Turnbough (29), and Farchaus et al.(11) who reported that EA1 is more frequently associated with vegetative cell material that has not been completely removed from spore preparations, while SAP is often associated both with cell material and in the supernatant of the preparation (22). Due to the similarity in size and sequence of these two proteins, further work is required to determine the specific identification of the 100 kDa band observed through protein chip analysis. Given our analysis used only supernatant preps at room temperature, our 100kDa bands are likely SAP. Further extraction would likely reveal higher concentrations of the combined proteins. Regardless, the presence of either of these proteins indicates that vegetative cell material is likely present in the Ultra Pure and 1X Wash preparations and may play a role in other biodetection assays.

ElectroChemiluminescence. The difference in Electrochemiluminescent assay response between Ultra Pure and 1X Wash (Figure 6) may be the result of increased EA1 or SAP protein concentration in the 1X Wash treatment samples due to purification treatments. The monoclonal reporter antibody used in this study's Electrochemiluminescent assay was raised against spores with some leftover vegetative material, and thus believed to cross-react with EA1. Therefore, if EA1 is present in a sample, it competes with the target *B. anthracis delta* Sterne spores bound to the polyclonal capture antibody (already bound to a magnetic dynabead) to attract and bind the reporter antibody and form the sandwich immuno-complex. This, in turn, leads to a decreased number of immuno-complexes being formed and anchored to the magnet in the BioVeris M1M's flow cell. When the flow cell is preconditioned with the assay buffer necessary to drive the Electrochemiluminescent assay reaction, the detector antibodies bound to EA1 are washed out of the flow cell, leaving only those immuno-complexes that formed properly and anchored to the magnet in the flow cell (via a magnetic dynabead) to yield a Electrochemiluminescent response. The further decreased response of Gamma Irradiated samples compared to the Ultra Pure and 1X Wash treatments may also be the result of poor immuno-complex formation. Changes to exosporium appearance have been previously noted following irradiation (23). Removal and/or partial destruction of epitopes (sites on the exosporium where antibodies bind to the spore) by irradiation treatment would greatly decrease assay response in an Electrochemiluminescent assay that utilizes a monoclonal reporter antibody whose binding options by nature are very specific and/or limited. Dang (7) also reported decreased enzymatic assay responses to *B. anthracis delta* Sterne spores after irradiation and proposed destruction of surface layer epitopes as cause for the decrease in assay response.

ELISA. The ELISA response across the concentrations tested is presented in Figure 7. The two antibodies used in the ELISA assay were the same as those used in the Electrochemiluminescent assay. In the ELISA assay, the EA1 or SAP protein once again competed with the target *B. anthracis delta* Sterne spores to attract and bind the detector antibody to form the sandwich immuno-complex, ultimately leading to a decrease in response. Numerous plate washes during the ELISA process removed a lot of malformed immuno-complexes, leaving fewer properly formed immuno-complexes to produce a response. As an alternate interferent, irradiation treatment led to a further decreased Optical Density response (and subsequent Limit of Detection) compared to the Ultra Pure and 1X Wash treatments. As was observed in the Electrochemiluminescent results, irradiation may remove or destroy antibody binding sites on the exosporium leading to a decrease in ELISA response (7). Our ELISA findings are consistent with Dang (7), who also measured decreases in ELISA response to irradiated samples, but interestingly enough, saw an increase in ELISA response to autoclaved spores and extracellular antigens. Conversely, Daupin (8) reported mixed results when using direct, indirect, and sandwich ELISA. Certainly direct comparison using identical deactivation and detection methods on the same virulent and non-virulent strains is warranted.

Our data has demonstrated response differences observed using total Protein, qPCR, ELISA, and Electrochemiluminescent assays. The identification of EA1/SAP proteins with the Protein 230 Labchip and removal of these proteins by gradient purification are demonstrated as important steps in quality antigen preparation. Gradient purification demonstrated a great improvement in spore sample quality in DNA and enzymatic assays through protein and extracellular DNA removal. Irradiation treatment may change spore surface characteristics that can lower enzymatic assay response yet increase DNA detection when analyzing untreated qPCR

samples. Assays that quantify *B. anthracis delta* Sterne using qPCR must consider the cellular versus extra-cellular disposition of sample DNA. Additionally, live versus dead cell contribution should be a first consideration when evaluating a material as a concentration reference.

Data is also presented in the literature demonstrating differences in assay detection based on purity, inactivation, and virulence (29,8,7) of *B. anthracis delta* Sterne. Uniform conformance testing and standardized protocols for post growth treatment of sporulated test materials are needed to bring the use of biological test materials into a uniform quality system.

ACKNOWLEDGEMENTS

The authors would like to thank Dan Carmany of Battelle and Darrel Menking for technical consultations, and Jude Height for critical review of this manuscript. We thank Corkey Smith and Janett Stein for assistance with library services. We would like to acknowledge Ngai Wong at the Defense Threat Reduction Agency for technical consultations and funding of this study.

REFERENCES

1. **Almedia, J.L., L. Wand, J.B. Morrow, and K.D. Cole.** 2006. Requirements for the development of *Bacillus anthracis* spore reference materials used to test detection systems, J, Res. Natl. Inst. Stand. Technol. 111:205-217.
2. **Almeida, J.L., B. Harper, and K.D. Cole.** 2008. *Bacillus anthracis* spore suspensions: determination of stability and comparison of enumeration techniques. J. Appl. Microbiol. 104:1442-1448.

3. **Anderson, D.O. and A.J. Mohr.** 2004. Abbreviated test report for technology readiness evaluation 2 for networked biological point detectors, WDTC-TR-03-134 (West Desert Test Center, U.S. Army Dugway Proving Ground, Dugway Utah) AD-B297339 UNCLASSIFIED.

4. **Belgrader, P., D. Hansford, G.T.A. Kovacs, K. Venkateswaran, R. Mariella Jr. F. Milanovich, S. Nasarabadi, M. Okuzumi, F. Pourahmadi, nd M.A. Northrup.** 1999. A minisonicator to rapidly disrupt bacterial spores for DNA analysis. Anal. Chem. 71, 4232-4236.

5. **Borthwick, K.A.J., T.E. Love, M.B. McDonnelland W.T. Coakley.** 2005. Improvement of immunodetection of bacterial spore antigen by ultrasonic cavitation, Anal. Chem. 77:7274-7245.

6. **Burns, M.J., G.J. Nixon, C.A. Foy, and N. Harris**. 2005. Standardization of data from real-time quantitative PCR methods evaluation of outliers and comparison of calibration curves. BMC Biotechnology 5:31, 2005.

7. **Dang, J.L., K. Heroux, J. Kearney, A. Arasteh, M. Gostomski, and P.A. Emanuel.** 2001. *Bacillus* spore inactivation methods affect detection assays. Appl. Environ Microbiol 67:8 3665-3670.

8. **Daupin, L.A., B.R. Newton, M.V. Rasmussen, R.F. Meyers, and M.D. Bowen.** 2008. Gamma irradiation can be used to inactivate *Bacillus anthracis* spores without compromising the sensitivity of diagnostic assays. Appl. Environ. Microbiol. 74:4427-4433.

9. **Department of Health and Human Services**, 2009. Bioterrorism Agents/Diseases, CDC, http://www.bt.cdc.gov/agent/agentlist-category.asp.

10. **Edmunds, J. M., P.J. Collett, E.R. Valdes, E.W. Skowronski, G.J. Pellar, and P.A. Emanuel**. 2009. Surface sampling of spores in dry-deposition aerosols. Appl. Environ. Microbiol. **75:**39-44.
11. **Farchaus, J.W., W.J. Ribot, M.B. downs, and J.W. Ezzell,** 1995. Purification and Characterization of the Major Surface Array Protein from the Avirulent *Bacillus anthracis* Delta Sterne-1, J. Bacteriol 177:9 2481-2489.
12. **Fouet, A., S. Mesnage, E. Tosi-Couture, P. Gounon, and M. Mock**. 1999. *Bacillus anthracis* surface: capsule and S layer. *J Applied Microbiol* **87**, 251-255.
13. **Fox, A., and 17 other attendees.** 2002. Report of the "Bioterrorism Workshop", J. Microbiol. Meth. 51:247-254.
14. **Fruchey, I.R. and P.A. Emanuel.** 2006. Market survey: biological detectors, ECBC-SP-019 (Edgewood Chemical Biological Center, Aberdeen Proving Ground, Md) AD-A456146. Unclassified
15. **Gilchrist, J.E., J.E. Campbell, C.B. Donnelly, J.T. Peeler, and J.M. Delaney. 1973.** Spiral plate method for bacterial determination, Appl. Microbiol. **25:**244-252.
16. **Grubb's Test for Outliers**, 2006. 1.3.5.17., NIST/SEMATECH e-Handbook of statistical methods, http://www.itl.nist.gov/div898/handbook/2006.
17. **Guide for the Selection of Biological Agent Detection Equipment for Emergency First Responders**, 2007. Vol. 1&2, DHS Guide 101-06 2nd Ed.
18. **Kournikakis, B., C. Bateman, and J.W. Cherwonogrodzky.**, 2000. Characterization of 21 strains of *Bacilus anthracis*. DRES-TM-2000-157 (Defence Research Establishment, Suffield Alberta) AD-A387399. UNCLASSIFIED.

19. **Laflamme, C., S. Lavigne, J. Ho, and C. Duchaine.** 2004. Assessment of bacterial endospore viability with flourescent dyes. J. Appl. Microbiol. 96:684-692.

20. **Luo, S. and A.J. Mohr.** 1997. Abbreviated test report for laser-induced fluorescence- data acquisition for agents of biological origin, DPG-TR-97-049-PT-1 (West Desert Test Center, Dugway Proving Ground, Utah) AD-B229960, UNCLASSIFIED.

21. **Luo, S. and A.J. Mohr.** 1998. Abbreviated test report for mass spectrometer biological profiling, DPG-TR-98-094-PT-4 (West Desert Test Center, Dugway Proving Ground, Utah) AD-B241-544, UNCLASSIFIED.

22. **Mesnage, S. E. Tosi-Couture, M. Mock, P. Gounon and A. Fouet,** 1997. Molecular characterization of the Bacillus anthracis main S-layer component: evidence that it is a major call-associated antigen. Mol Microbiol 23: 1147-1155.

23. **Philips, A.P., A.M. Campbell, and R. Quinn**. 1988. Monoclonal antibodies against spore antigens of *Bacillus anthracis*. FEMS Microbiol. Immunol. 47:169-178.

24. **Schumacher, W.C., C.A. Storozuk, P.K. Dutta, and A.J. Phipps.** 2008. Identification and characterization of *Bacillus anthracis* spores by multiparameter flow cytometry. Appl Environ Microbiol 74:16 5220-5223.

25. **Sequeira, A.** 2002 & 2003. Aptamer-enhanced biorecognition reagent system, (Omnisite Biodiagnostics Inc. Austin TX) https:/cbnet.apgea.army.mil/org/rda/od/odi/LIBRARY/pdf/b288845.pdf AD-B288845, AD-B307292, UNCLASSIFIED.

26. **Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. GartnerM.D. Provenzano. E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk.** 1985. Measurement of protein using Bicinchoninic Acid. Anal. Chem. 150;75-85.

27. **Stopa, P.J.,** 2000. The flow cytometry of *Bacillus anthracis* spores revisited. Cytometry 41:237-244.

28. **Stopa, P.J., S.A. Sincock, C.R. Johnson.** 2000. Evaluation of sensitive membrane antigen rapid test (SMART) tickets for the detection of biological materials, ECBC-CR-002 (Science and Technology Corp. Hampton, VA) AD-B252644. UNCLASSIFIED.

29. **Williams, D.D. and C.L. Turnbough, Jr.** 2004. Surface layer protein EA1 is not a component of *B anthracis* spores but is a persistent contaminant in spore preparations, J. Bacteriol 186;2 566-569.

30. **Winters, D.R., D.D. Martin, and B.G. Harper**. 2003. Abbreviated test report for the Defense Threat Reduction Agency laboratory report. WDTC-TR-02-077 (West Desert Test Center, Dugway Proving Ground, Utah) AD-B286531 UNCLASSIFIED.

584 TABLE 1. Data for Spiral Plate Colony Enumeration of *B. anthracis delta* Sterne Flask Growths.
585

Sample ID	Mean (CFU/mL)	Sample ID	Mean (CFU/mL)
UP 1	1.16×10^8	Irrad 1	1.70×10^6
UP 2	1.72×10^8	Irrad 2	1.00×10^7
UP 3	1.25×10^8	Irrad 3	8.41×10^6
1X 1	1.00×10^8		
1X 2	1.70×10^8		
1X 3	1.34×10^8		

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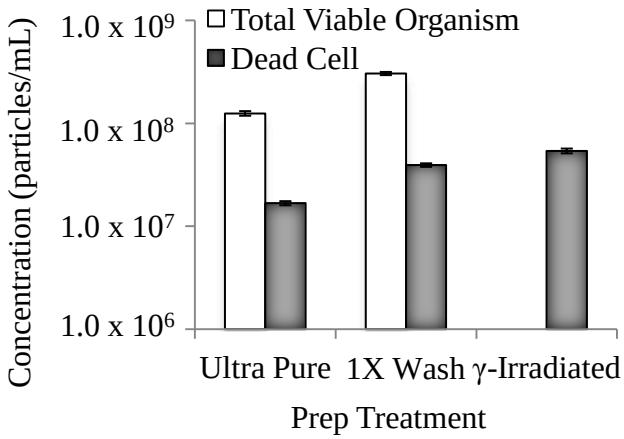


FIG. 1. MicroPRO Total Viable Organism and Dead Cell Assay Results, Mean and Standard Error (bars), for all Prepared *B. anthracis delta* Sterne Treatments. Ultra Pure and 1X Wash were enumerated using the Total Viable Organism (clear) and, Dead Cell (shaded) assays. Gamma Irradiated samples were enumerated with Dead Cell assay only. Data is presented for treatment mean and standard deviation.

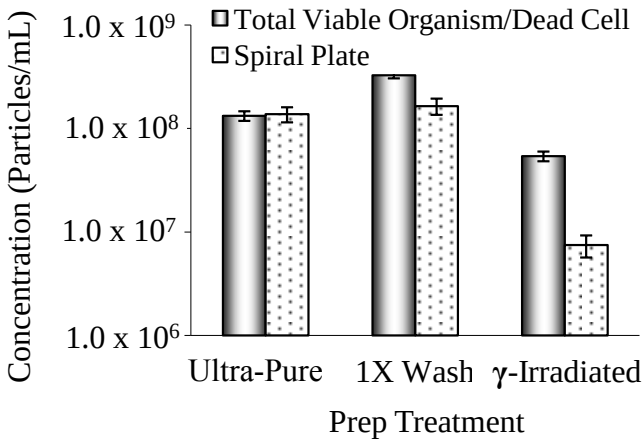
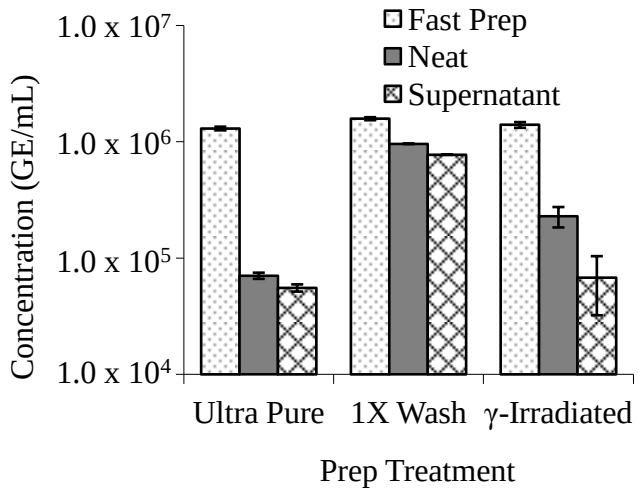


FIG. 2. Comparison of Mean values for *B. anthracis delta* Sterne Treatments from MicroPRO (clear) (Total Viable Organism and Dead Cell) and AP4000 (shaded) Analysis. MicroPRO Total Viable Organism analysis is represented for Ultra Pure and 1X Wash treatments, Dead Cell analysis is represented for Gamma Irradiated treatment.



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607 FIG. 3. Comparisons of qPCR Results for Growth Treatment and qPCR Sample Preparation
608 Treatments. FastPrep (dotted) represents bead-beaten pelleted re-suspension samples. Neat
609 samples (grey) are untreated (cellular and extra-cellular DNA) samples; Supernatant (crossed)
610 represent extra-cellular DNA, un-beaten samples. Bars are standard error.

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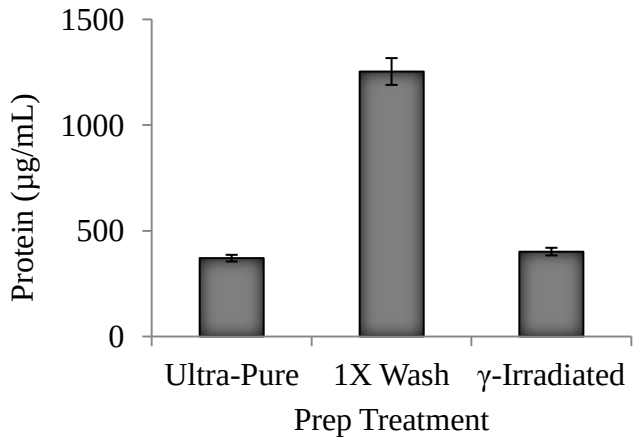


FIG. 4. Bicinchoninic Acid protein assay results for mean of each treatment of *B. anthracis delta* Sterne post growth treatments at sample concentration of 1 x 10^7 CFU/mL. Bars indicate standard error. The number of observations for Ultra Pure, 1X Wash, and Gamma Irradiation are 49, 25, and 30 respectively.

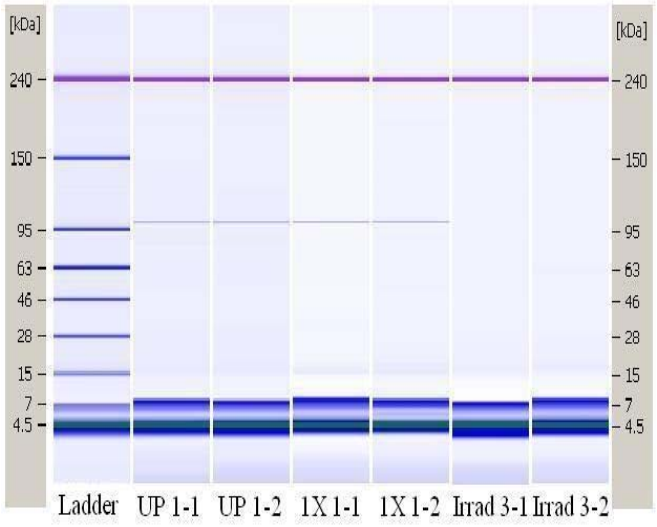
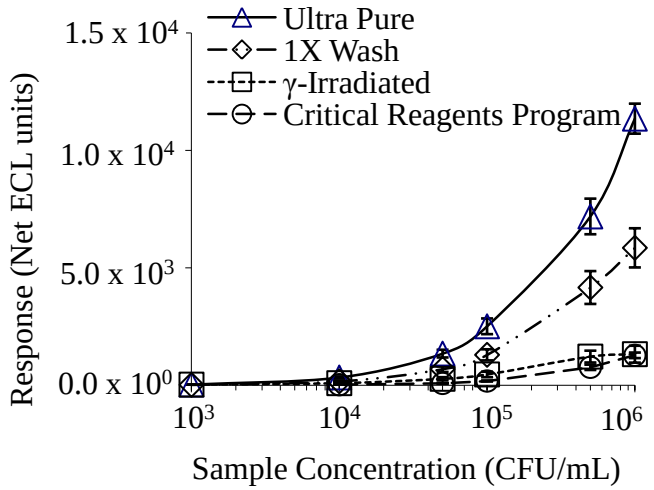


FIG. 5. Protein 230 LabChip results for Ultra Pure, 1X Wash and Gamma Irradiated post treatments. Bands at 97 kDa for Ultra Pure and 1X Wash samples represent protein concentrations of 6.9, 8.0, 7.0, and 7.5 ng/µL respectively.



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630 FIG. 6. Comparison of Electrochemiluminescent assay responses to the three *B. anthracis delta*
631 Sterne post growth treatments, Ultra Pure (Δ, n=26), 1X Wash (◊, n=24), Gamma Irradiated (□,
632 n=26), and the *B. anthracis delta* Sterne, lot AGD0000574, Critical Reagents Program reference
633 material (○, n=3). Sample means and standard error (bars) are shown.

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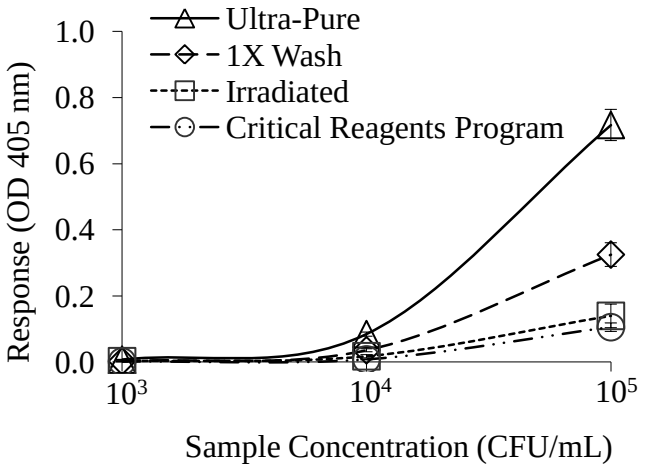

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638 FIG. 7. Responses of ELISA assay to the three *B. anthracis delta* Sterne treatments, Ultra Pure
639 (Δ, n=18), 1X Wash (◊, n=18), Gamma Irradiated (□, n=18), and the Critical Reagents Program
640 reference material (○, n=18). *B. anthracis delta* Sterne, lot AGD0000574, sample means and
641 standard error (bars) are shown. Each data set has a log-linear function with greater than 0.999
642 R^2 fit across concentrations tested.

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